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14. ABSTRACT
The main goal of the study was to test the hypothesis that a reduction in gene expression (i.e. gene repression) could induce gene silencing (i.e. relatively stable loss of gene expression) in breast cells. Silencing of a variety of tumor suppressor genes plays a major role in the initiation and progression of breast cancer and our ultimate goal is to determine if environmentally induced gene repression plays a role as a trigger for the silencing events. The purpose of the proposed work was to confirm or refute the hypothesis. The anticipated scope of the work was to test tumor suppressor promoters known to silence in breast cancer for repression-mediated gene silencing, but we soon realized that this scope was too ambitious for a one-year funding period. We therefore changed the scope somewhat to ensure that during the funding period we could at least test the basic principle of the hypothesis. This approach was successful and we have now demonstrated that gene repression can induce gene silencing in mammalian cells. Moreover, we have recently acquired functional tumor suppressor gene promoters that are silenced in breast cancer and can use remaining funds to test these promoters for repression-induced silencing.

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gene repression; gene silencing; environmental exposure; breast cancer

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INTRODUCTION

The main goal of the study was to test the hypothesis that a reduction in gene expression (i.e. gene repression) could induce gene silencing (i.e. relatively stable loss of gene expression) in breast cells. Silencing of a variety of tumor suppressor genes plays a major role in the initiation and progression of breast cancer and our ultimate goal is to determine if environmentally induced gene repression plays a role as a trigger for the silencing events. The purpose of the proposed work was to confirm or refute the hypothesis. The anticipated scope of the work was to test tumor suppressor promoters known to silence in breast cancer for repression-mediated gene silencing, but we soon realized that this scope was too ambitious for a one-year funding period. We therefore changed the scope somewhat to ensure that during the funding period we could at least test the basic principle of the hypothesis. This approach was successful and we have now demonstrated that gene repression can induce gene silencing in mammalian cells. Moreover, we have recently acquired functional tumor suppressor gene promoters that are silenced in breast cancer and can use remaining funds to specifically test these promoters for repression-induced silencing. A no-cost extension has been requested.

BODY

Our original plan was to obtain gene promoters for tumor suppressor genes known to be silenced in breast cancer cells, link these promoters to the selectable *HPRT* cDNA, transfect these promoters stably into *HPRT* deficient MCF-7 breast cancer cells, use environmental agents to repress promoter function (and thereby reduce HPRT protein levels), and then determine if reduced HPRT protein levels could persist in the absence of continued promoter repression. Such a result would demonstrate that gene repression could induce gene silencing in breast cells. It quickly became apparent, however, that this agenda was too ambitious for a variety of reasons including the relatively slow growth of the MCF-7 cells and the time required to clone and test the tumor suppressor gene promoters. My fear was that we would create the reagents necessary to test the hypothesis within a year, but not have sufficient time to create conditions under which we could actually conduct the test. I therefore made the decision to modify the scope of the proposed work to allow us to test the most important part of the hypothesis, which is that gene repression can induce gene silencing. The system that was developed and the results obtained are detailed below.

We used the tet-off system to create a model in which expression of a target gene could be specifically repressed; in this case by exposure to doxycycline (Dox), a tetracycline analog. The target gene in this model system remained the selectable human *HPRT* cDNA, which encodes a

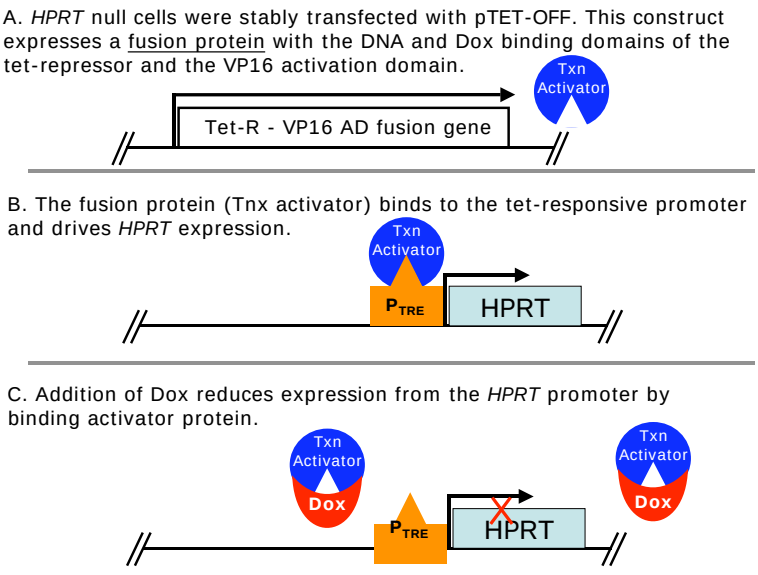

Figure 1. The tet-off system. Cells are transfected with a construct that expresses an activator protein (A) that binds to the tet response element (TRE) on a second construct that expresses HPRT cDNA (B). Addition of Dox to the medium leads to removal of the TRE protein from the promoter, and hence a reduction in gene expression (C).

protein that converts hypoxanthine and guanine to IMP and GMP, respectively. *HPRT* deficient cells can be selected by adding thioguanine (TG) to the culture medium because TG kills cells that express *HPRT*, whereas *HPRT* deficient cells can grow in the presence of TG. The Dox repressible construct expressing HPRT cDNA was transfected stably into a *Hprt* deficient mouse cell line termed DIF-6 that also contains the activator protein. Fig. 1 shows both constructs (1A and B) and how the system works to express HPRT, or to repress *HPRT* (1C) when Dox is added to the cell culture medium. Hence, Dox represses transcription of *HPRT* and removal of Dox from the medium leads to rapid restoration of *HPRT* expression.

The question we asked first was whether transient repression of *HPRT* transcription would lead to gene silencing, as predicted by the hypothesis. For these experiments, we treated three *HPRT* expressing transfectants containing the Dox repressible construct (HPRT 1, 3, and 4) for one week with 1 μM Dox. Following the one-week treatment, Dox was removed from the medium to allow *HPRT* expression to return, and then the cultures were exposed to TG. The results from a representative experiment are shown in Table 1. Although most cells died in the presence of TG after Dox was removed from the medium, which means these cells recovered *HPRT* expression, some cells became TG resistant cells due to Dox exposure at frequencies ranging from 10^{-3} to 10^{-4}. TG resistant clones were not observed in cultures that did not receive Dox treatment, with a single exception. Fig. 2 demonstrates that the frequency of TG resistant clones rose as a function of time that *HPRT* expression was repressed by Dox.

Table 1. Induction of phenotypic gene silencing via transient repression of *HPRT* cDNA with Dox[1].

Cell Line[2]	Treatment	Silencing Freq.[3]
HPRT 1[4]	untreated	9.8×10^{-6}
HPRT 1	1 μM Dox (7 days)	9.4×10^{-3}
HPRT 3	untreated	$< 4.5 \times 10^{-6}$
HPRT 3	1 μM Dox (7 days)	2.1×10^{-4}
HPRT 4	untreated	$< 4.4 \times 10^{-6}$
HPRT 4	1 μM Dox (7 days)	1.6×10^{-4}

1 Doxycycline (Dox) represses transcription of minimal CMV promoter by removing an activating protein.
2 Each cell line represents an independent transfectant.
3 Silencing frequencies represent the fraction of thioguanine (TG) resistant clones (see text for more detail).
4 This cell line gave rise to a single spontaneous TG resistant clone.

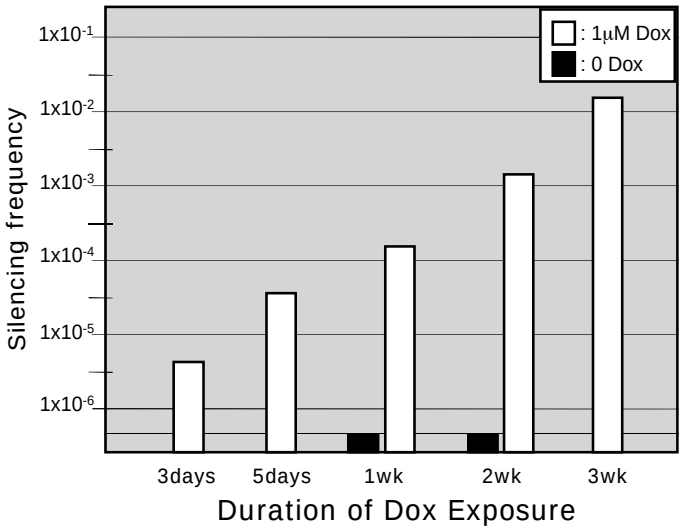

Figure 2. Silencing frequency increases as a function of Dox exposure. HPRT 3 cells (see Table 1) were exposed to Dox for the times indicated. After exposure, the cells were plated in the presence of TG to determine silencing frequencies. Cells exposed to no Dox (0 dox) for 1 and 2 weeks were also sampled, but neither plating yielded TG resistant clones.

The ability of the cells to grow into clones in the presence of TG after Dox was removed suggested that silencing occurred. However, this initial silencing event was short-lived because most TG resistant cells because most such clones were unable to sustain growth in TG in the absence of Dox. Nonetheless, approximately 10% of the TG resistant clones exhibited permanent resistance to TG (i.e., they continued to

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grow indefinitely in the presence of TG), which suggested that Dox exposure had induced long-term silencing in these clones. Preliminary work showed that loss of HPRT expression was not due to loss of expression of activator protein (data not shown), and therefore showed that the apparent silencing event was occurring at the *HPRT* locus itself. Further work was required, however, to distinguish silencing from mutational inactivation because of the relatively low percentage of cells that were stably TG resistant. The quickest way to distinguish bona fide mutational events from the silencing process is to measure reversion frequencies, which detect cells that reacquire expression of functional enzyme; silenced alleles often revert at high frequency, whereas mutant alleles revert at low frequency or not at all. Stable TG resistant clones induced by Dox exposure (from Table 1) were expanded and then plated in the presence of hypoxanthine and azaserine (HAz medium), which requires *HPRT* expression for cell survival. As shown in Table 2, Dox-induced TG resistant clones gave rise to spontaneous revertant clones at high frequencies (~ 10^{-3} to 10^{-2}) confirming that Dox-induced loss of gene expression was due to reversible silencing. Restoration of *HPRT* mRNA has been demonstrated using quantitative RT-PCR (data not shown). Significantly, treatment of the TG resistant cells cells with trichostatin A, a histone deacetylase inhibitor, dramatically increased the frequency of revertant cells (Figure 3), whereas 5-aza-dC, a DNA methylation inhibitor, had no effect (data not shown). This result demonstrated than an early step in gene silencing is histone deacetylation and that it does not require promoter region DNA methylation. The one spontaneous TG resistant clone (isolated from untreated HPRT 1 cells, Table 1) did not give rise to revertant cells, which demonstrated that this clone lost *HPRT* expression via a rare mutational event.

Table 2. Reversion Frequencies for TG resistant clones induced by Dox.

TG Cell Line[1]	Reversion Freq.[2]
HPRT 1-TG1[3]	< 0.4 X 10^{-6}
HPRT 1-Dox-TG2	5.1 X 10^{-3}
HPRT 1-Dox-TG3	2.5 X 10^{-3}
HPRT 3-Dox-TG1	1.8 X 10^{-2}
HPRT 3-Dox-TG2	1.2 X 10^{-2}
HPRT 3-Dox-TG1	7.6 X 10^{-3}
HPRT 3-Dox-TG2	1.6 X 10^{-3}

1 TG resistant cell lines derived from Table 1.
2 Reversion frequencies measured in HAz medium.
This cell line isolated from untreated culture.

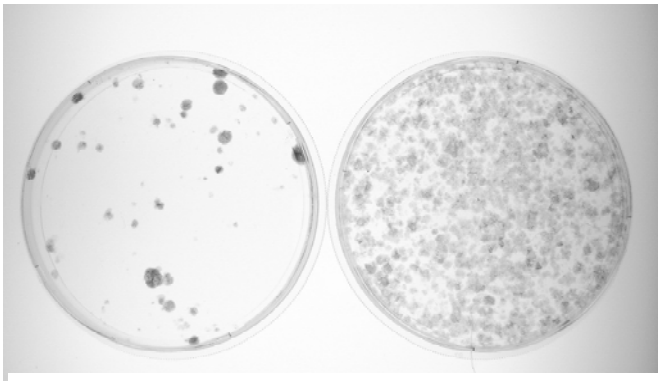

Figure 3. Induction of revertant cells with trichostatin A (TSA). A Dox-induced TG resistant subclone of the H3 cell line was exposed to 300 nM TSA for 24 hours and compared with untreated cells (Control) using the reversion assay (Fig. 2). An equal number of cells were plated in both dishes. The results show a significant induction of revertant cells via TSA treatment

In sum, the results can be interpreted as follows: Reversible gene repression is just that in the vast majority of cells. After the repressing agent (i.e. Dox) is removed from the medium, HPRT expression returns in most cells (>99.9%), as reflected by the inability of these cells to form clones in the presence of TG. In a subset of cells (< 0.1%) persistent gene repression consistent with gene silencing is observed after Dox exposure because these cells can form clones in the presence of TG. The majority of these initial TG resistant cells (approximately 90%) eventual lose the ability to grow in the presence of TG. This result indicates that the first

step in silencing is quite unstable. Nonetheless, cells that exhibit stable silencing of the *HPRT* construct can arise in approximately 10% of the TG resistant clones, thus demonstrating a second and more stable step in the silencing process. The second step is reversible by inhibiting histone deacetylation, which demonstrates that this chromatin modification occurs early in the silencing process. These results confirm that silencing is a multi-step process and provide the first system we are aware of in which silencing can be induced via a well-defined mechanism (i.e., gene repression).

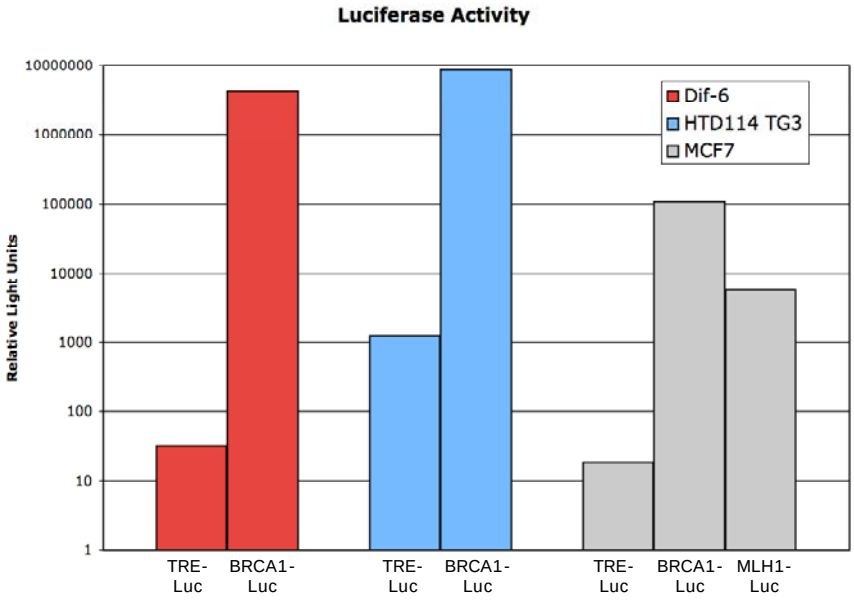

Figure 4. Demonstration of promoter activity in cell lines. The human BRCA-1 promoter linked to luciferase is shown to express in a mouse cell line (Dif-6), a human fibrosarcoma cell line (HTD114), and a human breast cancer cell line (MCF7). The MLH1 promoter was only tested in the MCG7 cells. The control TRE-Luc construct lacks a promoter. Note that the Y axis showing luciferase activity is on a log scale.

Finally, during the last year we have isolated or acquired functional promoters for the *BRCA-1*, *MLH1*, and E-cadherin tumor suppressor genes and have ligated these promoters to HPRT cDNA for eventual use in breast cell lines. Fig. 4 shows that these promoters are functional because they can drive expression of the luciferase reporter gene. (E-cadherin has not yet been tested.) Each promoter can be repressed by one or more environmental agents that have been linked to breast cancer, and it is our intention to use remaining funds to demonstrate that environmental repression can induce silencing following the general strategy described above. A no-cost extension has been requested.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that gene repression can lead to gene silencing in mammalian cells.
- Demonstrated that silencing is a multi-step process.
- Demonstrated that the earliest step in gene silencing is quite unstable.
- Demonstrated that the earliest step in gene silencing can progress to a more stable form.
- Demonstrated that an early step in gene silencing is histone deacetylation.
- Obtained functional tumor suppressor gene promoters for use in completion of the proposed work.

REPORTABLE OUTCOMES

- The data obtained from the Concept Award was use to apply for a three-year IDEA Award.
- We have created cell lines in which gene silencing can be triggered by adding Dox to the medium and the silencing process can be dissected and studied.

CONCLUSION

The main significance is that we have demonstrated that gene repression can trigger gene silencing. To the best of my knowledge, this is the first clear demonstration of a trigger for gene silencing; the experimental design is strongly suggestive that environmental repression can induce gene silencing. Thus we have provided the first experimental system in which gene silencing can be triggered and studied in mammalian cells. Additional work is anticipated to show similar effects with tumor suppressor promoters known to be silenced in breast cancer (see Figure 4). An understanding of how gene silencing is triggered can pave the way for preventing this process, which can thereby help to devise therapies to prevent breast cancer.

REFERENCES

N/A

APPENDICES

N/A

SUPPORTING DATA

Included above